COMPOSITIONS AND METHODS FOR ENHANCING IMMUNE RESPONSES MEDIATED BY ANTIGEN-PRESENTING CELLS

The disclosure of commonly-owned, co-pending U.S. Application Serial No. 08/299,285, is incorporated by reference herein.

FIELD OF THE INVENTION

The present invention relates to the field of vaccines and stimulation of acquired immunity. In particular, the present invention provides novel compositions designed to deliver specific antigens to antigen presenting cells and simultaneously deliver signals to those cells that produce a desired immune response.

BACKGROUND OF THE INVENTION

Several publications are referenced in this application by numerals in parenthesis in order to more fully describe the state of the art to which this invention pertains. Full citations for these references are found at the end of the specification. The disclosure of each of these publications is incorporated by reference herein.

The basis of acquired, specific immunity in an organism is the ability to discriminate between self and non-self antigenic substances. The mammalian immune system uses cell surface molecules known as the major histocompatibility complex (MHC) for discriminating between s lf from non-self. There are two classes of MHC molecules: Class I molecules are found on all nucleated

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cell types in the body; Class II molecul s are found
mainly on cells involved in producing immune responses.
Most specific immune responses are generated against
peptides or peptide derivatives associated with MHC
5   molecules.

The structure of MHC molecules is such that
they naturally bind small peptides, glycopeptides,
phosphopeptides, and the like. One important function of
MHC molecules is to bind peptides that are derived from
10   processed products of proteins expressed in cells
expressing the MHC molecules, and to transport these to
the cell surface for display to the immune system. In
this way, some MHC molecules function to expose the
immune system to peptides that are representative of
15   normal cellular proteins. This process occurs during
development, when self is learned, and continues
throughout the organism's lifespan. Different mechanisms
of immune tolerance prevent the organism from responding
to "self" peptides associated with MHC.

20   The introduction of non-self proteins into
cells results in the appearance of new and different
peptides in association with the MHC molecules; these are
recognized as "non-self," resulting in an immune
response. For example, viral infection of a cell will
25   result in the production of viral peptides expressed on
the surface of antigen-presenting cells in association
with MHC molecules (generally Class I MHC). Viral
peptides presented with MHC molecules at the cell surface
will often be recognized as foreign and an immune
30   response will be mounted. Autoimmune disease can occur
if tolerance to some self peptides is lost, or if immune
response is produced against viral or other foreign
proteins that cross react with normal peptides in the
host organism.

35   In the case of bacterial infections or other
insults from xternal sources, new proteins or compounds
enter the organism. Some cells involved in the immune

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r sponse are capable of phagocytosing foreign organisms
or proteins. These immune cells d grade (proc ss) the
protein products and the derived peptides are expressed
at the cell surface in association with MHC molecules,
5   where a specific adaptive immune response is generated
against novel non-self components. This activity is
called antigen processing and presentation and cells that
mediate this activity are called Antigen Presenting Cells
(APC's). Many different immune cell types, including
10   macrophages, dendritic cells, B cells, and other
associated cell types, perform this function.
        Antigen alone is often insufficient to produce
an immune response. Sometimes, antigen must be presented
with accompanying "signals" that are mediated by ligand-
15   receptor interactions between the APCs and the responding
lymphocytes or between these cells and soluble factors
that are present in the surrounding environment. The
soluble factors include cytokines and other mediators of
inflammation that are usually present at sites of
20   infections or insult (complement, kinins, other growth
and cytokine factors). The signals can be positive in
nature, resulting in lymphocyte proliferation and
generation of an adaptive immune response, or negative in
nature, resulting in apoptosis of responding lymphocytes
25   and perhaps immune tolerance to that antigen. Antigen
presentation often occurs in the presence of helper T
cells or other cell types that secrete arrays of
cytokines, which influence or determine the type of
immune response that is induced. At a cellular level,
30   specific immune responses are generated in a mixed
cellular environment that includes different types of
antigen presenting cells, helper T lymphocytes, other
types of regulatory cells, and the responding lymphocytes
(B cells for antibody responses and T cells for cellular
35   responses). Direct recognition of peptides by T cells
can also occur with some cell types, such as allografts,
where the allogeneic MHC is directly recognized as

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foreign.

    Antigen proc ssing and its impact on types of
immune responses to specific antigens. The mechanism by
which antigen is processed and presented and the
parameters that determine the types of immune responses
that are generated (antibody versus cellular) are at
present not well understood for many antigens. It is
believed that there are different classes of APCs that
can produce different types of immune responses. In
general, APC-induced responses to exogenous antigens that
are taken up by endocytosis are believed to be presented
to the immune system in the context of Class II MHC and
lead to recruitment of T helper cells that interact with
B cells and ultimately produce an antibody response. In
contrast, endogenous peptides from cells associate with
MHC Class I molecules and produce cellular activities
that include cytotoxic T lymphocytes (CTL) and Delayed
Type Hypersensitivity (DTH) T-cells. There are important
exceptions to these mechanisms. For example, many CTLs
reactive with exogenous peptides have been described, and
it is possible to generate CTLs to specific peptides that
have been added to *in vitro* cultures of immune cells.
    Other factors can determine the types of immune
responses that are generated. For example, the nature of
peptide association with MHC (either Class I or Class II)
is an important factor that influences types of immune
responses. In the case of Class I MHC molecules, there
are specific binding motifs for peptide association
(Rammensee et al, Ann. Rev. Imm. $\underline{11}$: 213, 1993). Binding
motifs have been established for H-2 K^d, K^k, D^d, and other
murine and human MHC. There are also parameters of
peptide sequence that determine affinity for class II
MHC. Thus, the types of peptides to which an individual
can mount an immune system response are in part
determined by the immunogenetic genotype and phenotype,
which establish the shape and structure of the MHC

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molecul s expressed by that individual.

In summary, the types of immune response that
are generated in an organism in response to antigenic
challenge is the result of a myriad of contributing
5   factors, including: the immunogenetic background of the
individual, prior sensitization to antigens, the route
and form of antigen exposure, age and gender of the
organism, and other factors. Almost all acquired immune
responses that involve specific T-cell recognition are
10   directed toward small peptides bound to the peptide
binding groove of MHC molecules, the obvious exception
being the response to superantigens. Cellular immune
reaction (T-helper reaction, CTL, DTH) to peptides bound
to MHC are usually generated through presentation of the
15   antigen to T cells by antigen-presenting cells (APCs).

Tumor Vaccines. Cancer cells express aberrant
molecules known as tumor-associated antigens. The immune
system has the potential to recognize such structures as
20   "foreign" and to mount specific immune responses against
them, so as to reject tumor cells in much the same way
that an allograft is rejected. This provides the basis
for interest in the development of active specific
immunotherapeutic (ASI) agents (cancer "vaccines") based
25   on cancer-associated antigens.

Early studies on rodent tumors induced by
chemical carcinogens, ultraviolet radiation, or viruses
showed induction of immunological rejection of secondary
tumor challenge. Subsequent studies on spontaneous
30   tumors showed that these animals were incapable of
inducing immune-mediated rejection of the tumor.
Although a large number of human tumor-associated
antigens have been characterized, most of these are also
expressed by some normal cells. Therefore, immunological
35   tolerance to such molecules makes it difficult to
stimulate respons s against such antigens. Moreover, it
is a concern that induction of strong immune responses

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against self mol cules may result in the development of
autoimmune disorders. Since tumor-specific antigens are
seldom detected in spontaneous cancers, approaches to
develop active specific immunotherapy for common cancers,
5   based on tumor-associated antigens, have been viewed with
pessimism.
          Nonetheless, interest in tumor immunology and
the development of ASI in particular has persisted.
There are at least four reasons for the current interest
10   in ASI approaches. First, cell-mediated immune responses
have been recognized as the key factor in immunological
rejection of cancer. T cells recognize processed
peptides in association with major histocompatibility
complex (MHC) molecules, so intracellular proteins can
15   give rise to peptide targets for cell-mediated responses.
Further, since antigen processing and presentation are
critical steps in T cell recognition, cancer-associated
alterations (in its post-translational processing or
levels of expression) of a self protein may result in
20   presentation of novel peptide fragments on cancer cells.
Secondly, tumor specific point mutations in certain genes
have been characterized in several animal and human
cancers. Some of these mutations generate novel peptide
fragments that bind MHC molecules resulting in the
25   production of new epitopes for recognition by T cells.
This process allows for the induction of specific immune
responses against cancer cells carrying such mutations.
Third, manipulation of immune responses using cytokines,
mutated antigens, and other means have sometimes resulted
30   in tumor rejection even in cases of tumors that express
weakly immunogenic antigens. Fourth, some individuals
with severe immunodeficiencies have a higher incidence of
tumors than the normal population, suggesting that the
immune system plays an important role in eliminating some
35   tumors.
          Various methods have been utilized for
stimulating general immune responses, especially for non-

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antigenic or weakly antigenic substances of interest.
For example, adjuvants, such as complete Freund's and
Ribi's, have long been used for this purpos . These
adjuvants comprise oily solutions containing components,
5   such as lipopolysaccharides that stimulate generalized
immune responses. It is believed that the oils surround
a water-soluble antigen, such as a peptide, thereby
protecting it from degradation in the body and
facilitating phagocytosis and passage through cell
10   membranes of antigen presenting cells.
        Another approach to stimulating the
immunogenicity of a weakly-antigenic peptide or protein
has been to couple the weak antigen to a carrier protein
that is known to be a good immunogen. Common carrier
15   proteins include keyhole limpet hemocyanin, fowl gamma-
globulin and bovine serum albumin. Alternatively, the
immunogencity of a weak antigen may be enhanced by
polymerizing it into large aggregates by way of cross-
linking agents, such as glutaraldehyde. Both these
20   methods rest on the notion that a weak antigen coupled to
a strong antigen will enhance the generalized immune
response. In a similar method, solid-phase resins and
peptide synthetic methods may be employed to synthesize a
peptide repeatedly, to form a highly-branched complex.
25   Again, the basis for this approach is to present the
antigen in very unusual (and very "non-self") context to
the immune system, to stimulate antibody production.
        In yet another approach, a weakly antigenic
protein or peptide is attached to a solid particle such
30   as a latex bead or resin. The purpose of this approach
is to enhance phagocytosis of the antigen by macrophages.
Additionally, peptides and proteins have been
encapsulated in liposomes to enhance passage through
membranes of antigen presenting cells, to enhance
35   phagocytosis and to stimulate generalized immune
responses b cause of the "non-self" characteristics of
the liposome carrier.

5   The approaches described above have met with varying degrees of success in stimulating the immunogenicity of weakly antigenic or non-antigenic substances. However, they provide only a generalized stimulation of immunity, and are not designed to target

10 specific populations of immune system cells (such as antigen presenting cells). A desired objective in effecting therapeutic intervention in various disease states is to provide a means for specifically targeting a protein or peptide to a population of antigen-presenting

15 cells and thereby stimulate those cells to internalize the antigen of interest and present it to the immune system in an effective, specific context. Insofar as it is known, such a system is not currently available.

20 **SUMMARY OF THE INVENTION**

   The present invention provides novel compositions and methods for delivering specific antigens to antigen-presenting cells, and simultaneously delivering signals to those cells that produce a desired

25 immune response. The compositions of the invention are sometimes referred to herein as "APC-targeted activating antigens."

   According to one aspect of the invention, these APC-targeted activating antigens, which elicit an immune

30 response mediated by an antigen-presenting cell, comprise at least one antigenic moiety functionally linked to at least one targeting moiety that binds specifically to a characteristic determinant on the antigen-presenting cell. For purposes of the present invention, the term

35 "functionally linked" is defined generally as linking of the moieties in such a way that each moiety retains its intended function. This is particularly relevant with respect to the targeting moiety, which is designed to bind to a characteristic determinant on the antigen-

40 presenting cell.

    Antigen-presenting cells contemplated for

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targ ting according to the pres nt invention include, but
are not limited to, monocytes, dendritic cells,
macrophages, B cells and some T cells. In preferred
embodiments of the invention, the characteristic

5   determinant on the selected APC is a cell surface
receptor and the targeting moiety of the APC-targeted
antigen is a ligand that binds to the receptor. It is
particularly preferred that the cell surface receptor be
an immunomodulatory receptor. Suitable cell surface

10 receptors include, but are not limited to, C5a receptor,
IFNγ receptor, CD21 (C3d) receptor, CD64 (FcγRI) receptor,
receptor, and CD23 (FcεRII) receptor.

One exemplary APC-targeted antigen of the
invention is designed to bind to the C5a receptor, and

15 the targeting moiety is a C5a receptor ligand, which is
preferably a peptide analog of C5a corresponding to the
C-terminal 10 residues of C5a. Another exemplary
composition of the present invention is designed to bind
to the IFNγ receptor, and comprises a targeting moiety

20 which is a IFNγ receptor ligand, preferably a peptide
analog of IFNγ corresponding to the N-terminal 39
residues of IFNγ.

The antigenic moiety of the APC-targeted
antigens of the invention can comprise essentially any

25 antigenic substance, including, but not limited to,
peptides and proteins, glycopeptides and glycoproteins,
phosphopeptides and phosphoproteins, lipopeptides and
lipoproteins, carbohydrates, nucleic acids and lipids.
The APC-targeted antigens can comprise more than one

30 antigenic moiety, and likewise can comprise more than one
targeting moiety. Moreover, these moieties can be
functionally linked in several fashions. For instance,
if "T" represents a targeting moiety, and "Ag" represents
an antigenic moiety, the APC-targeted antigens of the

35 present invention may be oriented as follows:

Ag - T;

T - Ag;

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$$T_1 - Ag - T_2;$$

$$T_1 - [Ag]_n - T_2 \text{ (wherein } [Ag]_n \text{ represents a}$$
multiplicity of antigens.

Examples of the general formulas set forth
5   above include:

Ag - C5a agonist peptide;

IFNγ peptide - Ag;

IFNγ peptide - $[Ag]_n$ - C5a agonist peptide.

10   According to other aspects of the present
invention, methods are provided for using the APC-
targeted antigens of the invention. These include
methods of activating an antigen-presenting cell with a
targeting ligand and methods of eliciting an antigen
15   presenting cell-mediated immune response in a subject in
which such a response is desired. General methods of
immunizing or vaccinating a patient requiring such
treatment, methods of treating a tumor, and methods for
producing antibodies specific for a pre-determined
20   antigen for use as research tools or for diagnostic
purposes are also contemplated to be within the scope of
the present invention.

The numerous features and advantages of the
compositions and methods of the present invention are
25   described more fully in the detailed description set
forth below.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph illustrating the antibody titer
30   produced in mice immunized with the indicated peptide
constructs, as determined by radioimmunoassay, and shows
the relationship between the amount of ^{125}I-goat anti-mouse
antibody bound vs. the dilution factor of mouse sera
which had been incubated in microtiter wells coated with
35   the MUC1 epitope peptide.

Figure 2 is a graph illustrating the increase in

5　antibody titer in the sera of mice collected either
before (pre) or after immunization with peptides 3
(YKQGGFLGLYSFKPMPLaR) (SEQ ID NO:2) and 4
(YSFKPMPLaRKQGGFLGL) (SEQ ID NO:5) as determined by
radioimmunoassay and shows the relationship between the
10　amount of ^{125}I-goat anti-mouse antibody bound and the
dilution factor of mouse sera which had been incubated in
microtiter wells coated with MUC1 epitope peptide. Note
that peptides 3 and 4 comprise two moieties, a targeting
ligand and an antigen to which an immune response is desired.

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Figure 3 is a graph illustrating the titers of
antibody classes and subclasses produced in mice
following immunization with peptide 3
(YKQGGFLGLYSFKPMPLaR) (SEQ ID NO:2) as determined by
20　ELISA using rabbit anti-mouse IgA, IgG1, IgG2a, IgG2b,
IgG3, and IgM, followed by goat anti-rabbit conjugated to
peroxidase and detected using p-nitrophenyl phosphate
cleavage monitored at 405 nm.

25　Figure 4 is a graph illustrating the specificity of
binding of the antibody subclasses in sera from mice
immunized with peptide 3 (YKQGGFLGLYSFKPMPLaR) (SEQ ID
NO:2) as determined by ELISA using binding to microtiter
wells coated with MUC1 epitope peptide and detection with
30　rabbit anti-mouse IgG2a, IgG2b, or IgM followed by
incubation with goat anti-rabbit conjugated to peroxidase
and detected using p-nitrophenyl phosphate cleavage
monitored at 405 nm.

35　**DETAILED DESCRIPTION OF THE INVENTION**

A major obstacle in the development of vaccines
and other immunostimulatory agents is the inability of
some antigens to be readily taken up and processed by
antigen presenting cells. Uptake of antigens by APCs is
40　an essential step for stimulating an effective immune
response, sinc the immune system recognizes the antigen

only after it has been processed by the APC and presented
on the surface of the APC in conjunction with the major
histocompatibility complex (MHC).

5   It is known that APCs, including dendritic
cells, monocytes, macrophages and B cells, possess
functional receptors for numerous molecules that modulate
the immune response. It has now been discovered in
accordance with the present invention that ligands which
bind to these receptors can be conjugated to weakly
10   immunogenic antigens for example, as a way of delivering
antigens to the antigen presenting pathway of the APC and
simultaneously activating the antigen presenting capacity
of the APC. Thus, these conjugates bind to a receptor on
the APC surface, transduce a biological signal, and are
15   internalized by the APC. The antigenic moiety of the
conjugate is thereby delivered to the antigen presenting
pathway of the APC along with the simultaneous activation
of the APC.

The above-described conjugates are sometimes
20   referred to herein as "molecular adjuvants" or "APC-
targeted activating antigens." The APC-targeted
activating antigens of the invention are designed to
elicit immune responses mediated by one or more types of
antigen presenting cells. Accordingly, an APC-targeted
25   activating antigen comprises at least one antigenic
moiety linked to a targeting and activating moiety that
binds specifically to at least one characteristic
determinant on the selected antigen presenting cell type.
This binding is followed by internalization of the APC-
30   targeted antigen and results in presentation of the
antigen moiety on the surface of the APC. For purposes
of the present invention, the term "antigenic moiety" may
refer to any substance to which it is desired that an
immune response be produced. The selected antigenic
35   moiety may or may not be capable of eliciting an immune
response by conventional means.

The term "determinant" is used herein in its

broad sense to denote an element that identifies or
determines the nature of something. When used in
reference to an antigen presenting cell, "determinant"
means that site on the antigen presenting cell which is
5   involved in specific binding by the targeting ligand
moiety of the molecular adjuvant of the invention.

The expression "characteristic determinant" as
used herein, signifies an epitope (or group of epitopes)
that serves to identify a particular population of
10   antigen presenting cells and distinguish it from other
antigen presenting cell populations. Cell-associated
determinants include, for example, components of the cell
membrane, such as membrane-bound proteins or
glycoproteins, including cell surface antigens, histo-
15   compatibility antigens or membrane receptors.

The expression "specific binding", as used
herein refers to the interaction between the targeting
ligand moiety and a characteristic determinant on the
antigen presenting cell population sought to be activated
20   in accordance with this invention, to the substantial
exclusion of determinants present on other cells.

Certain exemplary compositions of the invention
have been synthesized, and have been shown to elicit APC-
mediated immune responses in accordance with the
25   mechanisms described above. For example, antigenic
epitopes have been conjugated to the amino-terminal end
of a C5a decapeptide agonist capable of binding to C5a
receptors present on the surface of many APCs. Mice that
were inoculated with an epitope of human MUC1 (a cell
30   surface-associated mucin) conjugated to such a C5a
agonist exhibited pronounced antibody titers against the
MUC1 epitope, including high titers of specific
antibodies with isotypes IgG2a and IgG2b. Mice that were
inoculated with (1) MUC1 epitope alone, (2) C5a agonist
35   alone, (3) unconjugated MUC1 epitope and C5a agonist
together, or (4) C5a agonist conjugated to MUC1 epitope
in a manner in which the biological activity of the C5a

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moiety was blocked, did not express a significant
specific immune response. These results are described in
greater detail in Example 1. Similar results were
observed with conjugates of C5a agonist to a 12 kDa
5   polypeptide, serum amyloid A (SAA), as described in
greater detail in Example 2. These data tend to
demonstrate the feasibility of the invention, which is to
use receptor-binding ligands as a way to deliver antigens
to APCs, with the simultaneous activation of APCs by the
10   ligand moiety.

As described in greater detail below, the C5a
receptor is only one of many receptors expressed on APCs.
This invention encompasses the use of various ligands
with selectivity to other receptors that mediate signal
15   transduction events in the APCs, to be used alone or in
conjunction with C5a agonists to influence the nature of
immune response generated, i.e., humoral, cellular, Th1,
Th2, and the like. Vaccines and other immunotherapeutic
agents can be prepared with an array of such targeting
20   moieties that serve to target the antigen moiety to a
specific population of APCs and simultaneously activate
these and other cells involved in various immune
modulatory pathways.

The detailed description below sets forth
25   preferred embodiments for making and using the targeted
antigens of the present invention. To the extent that
specific compounds and reagents are mentioned, these are
for the purposes of illustration, and are not intended to
limit the invention. Any biochemical, molecular or
30   recombinant DNA techniques not specifically described are
carried out by standard methods, as generally set forth
for example, in Ausubel et al., "Current Protocols in
Molecular Biology," John Wiley & Sons, Inc., 1995.

35   I. Preparing APC-Targeted Activating Antigens
         A.   Selection of Components
             Antigen presenting cells have various receptors

on their surfaces for known ligands. Binding of ligands
to these receptors results in signal transduction events
that stimulate immune or tolerance responses. Many of
these receptors are known to internalize and recycle in
5   the cell. Others are suspected of doing the same. As
such, these receptors are ideal targets for delivering
antigens and activation signals simultaneously to APCs.

        As discussed above, APCs include several cell
types including macrophages, monocytes, dendritic cells,
10   B cells, some T cells and other poorly characterized cell
types. It is believed that these different classes of
APCs can produce different types of immune responses.
Accordingly, by targeting a receptor prevalent on a
specific population of APCs, a particular desired immune
15   response may be favored. An exemplary list of receptors
contemplated for targeting in the present invention, and
the rationale for their selection, is set forth below.
These APC receptors are particularly appropriate for use
in the present invention based on the following criteria:
20   they are receptors expressed on APCs; the receptors are
internalized upon binding of ligand; the receptors can
transmit signals in the cells that influence antigen
processing and presentation by these cells; some of the
receptors are believed to be involved in signaling Th1
25   type cellular responses, whereas others are predicted to
generate Th2 type humoral responses. The list set forth
below is not exhaustive, but merely representative of the
type of targeted receptors preferred in practicing the
present invention. Other receptors, or other cell-
30   surface characteristic determinants on antigen presenting
cells may also be used as targets for the targeted
antigens of the present invention. The receptor or other
characteristic determinant need not be directly involved
in the immune response.
35   **C5a receptor.** This receptor is preferred for
use according to the present invention. It is present on
PMNs, macrophages, dendritic cells, smooth muscle cells

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and some mast cells. A number of biological activities
have been ascribed to C5a, mostly associated with
inflammatory and immune responses. According to a
preferred embodiment, this invention relies on the
5   capability of C5a, as a targeting ligand, to specifically
bind to its cognate receptor, so as to activate antigen
presenting cells, including macrophages, monocytes and
dendritic cells, through a G protein-mediated signal
transduction pathway. Subsequent to signal transduction,
10   the receptor/ligand complex is internalized. In the case
of dendritic cells, C5a has been shown to induce a Th1
type response.

       IFNγ receptor. The interferon γ receptor is
expressed on macrophages, monocytes, dendritic cells,
15   other APCs, some B cells, fibroblasts, epithelial cells,
endothelium, and colon carcinoma cells. IFNγ binding to
its receptor induces macrophage and dendritic cell
activation, B cell differentiation, and expression of MHC
class I and class II molecules in many cell types. The
20   receptor is involved in signal transduction pathways.
IFNγ is mainly produced in the body by activated T cells,
particularly during the generation of Th1 type response.
It is also produced by CD8+ cytotoxic T lymphocytes
following recognition of antigen associated with MHC
25   class I and by natural killer cells stimulated with TNFα
and microbial products (**Barclay et al. 1993,**).

       CD 21 (C3d receptor). CD 21 is the receptor
for the C3d complement fragment. It is a receptor for the
Epstein-Barr virus and may be an important interferon α
30   receptor (**Barclay et al.,** supra). CD 21 is expressed on
B cells, follicular dendritic cells, other APCs,
pharyngeal and cervical epithelial cells, and some
thymocytes. It is involved in activation and
proliferation of B cells through a signal transduction
35   mechanism and it has been associated with increases in
antigen presentation activities by those cells.

       CD 64 (FcγRI rec ptor). CD 64 is a high

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affinity receptor for IgG, the only known receptor that binds monomeric IgG (**Barclay et al**, *supra*). This receptor is found on macrophages, monocytes and other immune cell populations treated with IFNγ. The IgG$_1$

5   binding site resides in the CH2 domain. IFNγ strongly upregulates expression of this receptor, which is the primary receptor involved in antibody-dependent cell mediated cytotoxicity reaction, and phagocytic activity by these cells.

10   **CD 23 (FcϵRII receptor)**. CD 23 is a low affinity receptor for IgE (not related to the high affinity IgE receptor found on basophils and mast cells). It is found on some B cell populations, macrophages, eosinophils, platelets, and dendritic cells (Barclay et

15   al, *supra*). CD 23 mediates IgE dependent cell mediated cytotoxicity and phagocytosis by macrophages and eosinophils, and binding of IgE immunocomplexes increases the efficiency of antigen processing and presentation by some APCs, through a signal transduction mechanism that

20   includes the p59 fyn tyrosine kinase. The ligand for CD 23 is the Cϵ3 domain of IgE.

            As mentioned above, the APC-targeted antigens of the present invention comprise at least one antigenic moiety and at least one targeting moiety. The targeting

25   moiety can be derived from naturally-occurring ligands for a selected receptor on an APC, or analogs and derivatives of such ligands. For instance, the C5a receptor is a preferred receptor for use in practicing the present invention. Naturally-occurring C5a can be

30   utilized as the targeting moiety in the APC targeted activating antigens of the invention. However, native C5a is not preferred for use as the targeting moiety as it induces a myriad of pro-inflammatory responses which may have undesirable side effects. In particularly

35   preferred embodiments of the invention, C-terminal C5a agonist analogs capable of C5a receptor binding and signal transduction in a response selective manner are

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5   utilized. Such analogs are described in detail in
       commonly-owned U.S. Application Serial No. 08/299,285,
       the entire disclosure of which is incorporated by
       reference herein.

              An exemplary C5a C-terminal decapeptide agonist
10   preferred for use in the present invention is:

                    YSFKPMPLaR   (SEQ ID NO:1)

       This decapeptide is a potent agonist of naturally
       occurring C5a, and is preferred to naturally occurring
       C5a because its small size contributes to ease of
15   synthesis and solubility. Moreover, these
       conformationally biased peptides are stable toward serum
       carboxypeptidase digestion, express a level biological
       selectivity, and have been shown to be non-toxic in high
       concentrations in athymic mice.

20   Peptide analogs of naturally-occurring
       interferon γ are also contemplated for use in the present
       invention. Peptides corresponding to the amino terminal
       39 amino acids of IFNγ have been shown to compete for
       binding with native IFNγ. Antibodies against this domain
25   block biological activity, and removal of the first 10
       amino terminal residues eliminates biological activity.
       This suggests that binding of IFNγ to its cognate
       receptor is mediated by this portion of the molecule.
       Accordingly, peptides based on this domain are
30   contemplated to be of use for delivering antigens to APCs
       expressing IFNγ receptors. In this regard, it should be
       noted that human and mouse IFNγ are absolutely species
       specific in binding and activity. Consequently, for
       stimulating APC-mediated immune responses in mice, the
35   mouse peptides will be utilized, and the human peptide
       will likewise be utilized for stimulating APC-mediated
       immune responses in humans. The mouse IFNγ 39 amino acid
       peptide analog is composed of the following sequence:

       HGTVIESLESLNNYFNFFGIDVEEKSLFLDIWRNWQKDG   (SEQ ID NO:3)

40   The human IFNγ 39 amino acid peptide analog is composed
       of the following sequence:

5   QDPYVKEAENLKKYFNAGHSDVADNGTLFLGILKNWKEE   (SEQ ID NO:4)

Another ligand contemplated for use in the
present invention is the C3dG component of complement.
This component is a 348 residue fragment derived by
proteolytic cleavage from the C3b precursor (residue 955-

10   1303 of C3; Swissprot accession p01024). C3dG can be
converted to C3d (residues 1002-1303) and C3g (residues
955-1001). C3dG and C3d remain associated with non-
activator surfaces and serve as opsonins for phagocytosis
by macrophages and other antigen presenting cells. Cd 21

15   is the C3dG and C3d receptor.

The above-listed ligands exemplify the type of
ligand preferred for practice of the present invention.
However, it will be appreciated by those skilled in the
art that other ligands may be utilized as the targeting

20   moiety of the APC-targeted antigens of the invention.
These include ligands that are already known in the art,
as well as ligands that may be discovered and developed
henceforth. Antibodies or antibody fragments also may be
used to target APC-specific cell surface antigens.

25   The type of antigen that can be chosen as the
antigenic moiety in the present invention can be any
peptide, polypeptide or derivative thereof for which an
immune response or antibody production is desired. These
include but are not limited to, peptides, polypeptides

30   (i.e. proteins) and derivatives thereof, such as
glycopeptides, phosphopeptides and the like. Synthetic
peptide and polypeptide derivatives or analogs, or any
other similar compound that can be conjugated to a
receptor-targeting moiety can be used in the present

35   invention. Moreover, these peptides, proteins and
derivatives may comprise single epitopes or multiple
epitopes for generating different types of immune
responses. Indeed, if an entire protein is conjugated to
a targeting moiety, this protein is likely to comprise

40   numerous epitopes, which may vary depending upon the
solvent conditions and their effect on secondary and

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tertiary structure of the protein. Carbohydrates,
nucl ic acids and other non-protein substances also may
be used as the antigenic moiety. Methods are available
in the art for conjugating these substances to the
5   peptide or protein targeting moiety.

          In preferred embodiments of the invention, the
antigenic moiety comprises agents that are weakly
antigenic or non-antigenic under currently available
immunization conditions. Many tumor-associated antigens
10   fall into this category, because the antigens also are
expressed by normal cells. Therefore, immunological
tolerance to such molecules makes it difficult to
stimulate responses against such antigens. Other
proteins that fall into this category include naturally
15   occurring proteins from one species (e.g., human) for
which it would be desirable to produce antibodies in
another species but which are recalcitrant to antibody
generation in the other species.

          One well-characterized tumor antigen is a cell
20   surface-associated mucin that is highly overexpressed and
differentially glycosylated by different adenocarcinomas,
including breast, pancreas, lung and prostate carcinomas.
Aberrant glycosylation of MUC1 by adenocarcinomas results
in the addition of some blood group carbohydrate antigens
25   to this core protein and the exposure of epitopes which
have been detected by monoclonal antibodies on the core
protein that are not exposed on forms of this protein
produced by normal epithelial cells. A full-length cDNA
sequence of human mucin-1 (MUC1) revealed an encoded
30   protein with an average length of approximately 1200
amino acids (depending on the length of the tandem repeat
allele) with several obvious domains: an amino terminal
signal peptide; a large domain made up of multiple
identical 20 amino acid tandem repeats flanked by several
35   repeats that contain degenerate sequences; a hydrophobic-
spanning domain of 31 amino acids; and a cytoplasmic
domain of 69 amino acids at the carboxyl terminus.   The

5   most well-characterized tumor associated epitopes
       described to date for MUC1 are found in the tandem repeat
       domain. These include carbohydrate structures and
       protein structures. MUC1 tumor associated epitopes are
       well characterized, and thus have been proposed to be
10   used for the production of tumor vaccines using
       conventional methods. Exemplary compositions of the
       present invention comprise MUC1 epitopes, such as those
       set forth below, as the antigenic moiety of the APC-1
       targeted antigens of the invention, to demonstrate the
15   potential of the present invention as potent tumor
       vaccines.
              MUC1 epitope predicted to bind to class I
       molecules of the H-2k^b type has sequence homology to the
       juxtamembrane region of MUC1;
20   YKQGGFLGL   (SEQ ID NO:6)
              MUC1 tandem repeat has the sequence:
                GVTSAPDTRRAPGSTAPPAH   (SEQ ID NO:7)

              The components comprising the APC-targeted
25   antigens of the invention can be made separately, then
       conjugated. For example, a small peptide analog, such as
       the above-described C5a agonists, may be produced by
       peptide synthetic methods, and conjugated to a protein
       which has been purified from naturally occurring
30   biological sources. Alternatively proteins engineered
       for expression via recombinant methods may be used.
       Additionally, targeted antigens comprising peptide
       components (i.e., a peptide antigenic epitope conjugated
       to a peptide receptor ligand) can be synthesized in
35   tandem by peptide synthetic chemistry according to known
       methods and as described in greater detail below.
       Finally, targeted antigens of the invention comprising
       two larger polypeptide moieties (i.e., a large
       polypeptide antigen linked to a large ligand) can be made
40   by recombinant techniqu s. For example, DNA molecules
       encoding both components can be ligated together by

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recombinant means, then expressed as the conjugated
fusion prot in. Methods of making these different types
of compositions are set forth in greater detail below.

5   B.   Peptides
          Oligopeptides required for the present
invention may be prepared by various synthetic methods of
peptide synthesis via condensation of one or more amino
acid residues, in accordance with conventional peptide
10   synthesis methods. Preferably, peptides are synthesized
according to standard solid-phase methodologies, such as
may be performed on an Applied Biosystems Model 430A
peptide synthesizer (Applied Biosystems, Foster City,
CA), according to manufacturer's instructions. Other
15   methods of synthesizing peptides or peptidomimetics,
either by solid phase methodologies or in liquid phase,
are well known to those skilled in the art. When solid-
phase synthesis is utilized, the C-terminal amino acid is
linked to an insoluble resin support that can produce a
20   detachable bond by reacting with a carboxyl group in a C-
terminal amino acid. One preferred insoluble resin
support is p-hydroxymethylphenoxymethyl polystyrene (HMP)
resin. Other useful resins include, but are not limited
to: phenylacetamidomethyl (PAM) resins for synthesis of
25   some N-methyl-containing peptides (this resin is used
with the Boc method of solid phase synthesis; and MBHA
(p-methylbenzhydrylamine) resins for producing peptides
having C-terminal amide groups.
          During the course of peptide synthesis, branched
30   chain amino and carboxyl groups may be
protected/deprotected as needed, using commonly-known
protecting groups. In a preferred embodiment, N^{α}-amino
groups are protected with the base-labile 9-
fluorenylmethyloxycarbonyl (Fmoc) group or t-
35   butyloxycarbonyl (Boc groups). Side-chain functional
groups consistent with Fmoc synthesis may be prot cted
with the indicat d protecting groups as follows:

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arginine (2,2,5,7,8-pentam thylchroman-6-sulfonyl);
asparagine (O-t-butyl ester); cysteine glutamine and
histidine (trityl); lysine (t-butyloxycarbonyl); serine
and tyrosine (t-butyl). Modification utilizing

5   alternative protecting groups for peptides and peptide
derivatives will be apparent to those of skill in the
art.

C. Proteins

10   Full-length proteins for use in the present
invention may be prepared in a variety of ways, according
to known methods. Proteins may be purified from
appropriate sources, e.g., human or animal cultured cells
or tissues, by various methods such as gel filtration,

15   ion exchange chromatography, reverse-phase HPLC and
immunoaffinity purification, among others. However, due
to the often limited amount of a protein present in a
sample at any given time, conventional purification
techniques are not preferred in the present invention.

20   The availability of nucleic acids molecules
encoding a protein enables production of the protein
using *in vitro* expression methods known in the art. For
example, a cDNA or gene may be cloned into an appropriate
in vitro transcription vector, such a pSP64 or pSP65 for

25   *in vitro* transcription, followed by cell-free translation
in a suitable cell-free translation system, such as wheat
germ or rabbit reticulocytes. *In vitro* transcription and
translation systems are commercially available, e.g.,
from Promega Biotech, Madison, Wisconsin or BRL,

30   Rockville, Maryland.

         Alternatively, according to a preferred
embodiment, a selected peptide or protein may be produced
by expression in a suitable procaryotic or eucaryotic
system. For example, a DNA molecule, encoding a peptide

35   or protein component of the invention, or an entire
composit targeted antigen of the invention, may be
ins rted into a plasmid vector adapted for expression in

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a bacterial cell, such as *E. coli*, or into a baculovirus
vector for expression in an insect cell. Such vectors
comprise the regulatory elements necessary for expression
of the DNA in the host cell, positioned in such a manner
5   as to permit expression of the DNA in the host cell.
Such regulatory elements required for expression include
promoter sequences, transcription initiation sequences
and, optionally, enhancer sequences.

            A peptide or protein produced by gene
10   expression in a recombinant procaryotic or eucaryotic
system may be purified according to methods known in the
art. In a preferred embodiment, a commercially available
expression/secretion system can be used, whereby the
recombinant protein is expressed and thereafter secreted
15   from the host cell, so as to be readily purified from the
surrounding medium. If expression/secretion vectors are
not used, an alternative approach involves purifying the
recombinant protein by affinity separation, such as by
immunological interaction with antibodies that bind
20   specifically to the recombinant protein. Such methods
are commonly used for isolating peptides and proteins.

            D.   Linking Separately-Made Proteins
                  and/or Peptides
25   In an alternative embodiment, protein and/or
peptide components of the invention are synthesized
separately, then conjugated using standard methods known
by those skilled in the art. For example, a synthetic
peptide may be chemically coupled to a protein using *m*-
30   maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBF). This
reagent cross-links amino- and carboxy-terminal thiol
groups in the peptide with lysine side chains present in
the protein. Alternatively, a synthetic peptide may be
coupled to a protein using glutaraldehyde, a common
35   cross-linking agent. Another method for chemically
coupling a peptide to a protein is through the use of
carbodiimide and 1-(3-dimethylaminopropyl)-3-

5 ethylcarbodiimide methiodide (EDC). As described in greater detail in Example 2, this method was used to conjugate a C5a C-terminal decapeptide analog to serum amyloid A (SAA). Methods for joining two proteins together are also available.

10 The peptides or proteins of the invention, prepared by the aforementioned methods, may be analyzed according to standard procedures. For example, they may be subjected to amino acid sequence analysis, mass spectra analysis or amino acid compositional analysis

15 according to known methods.

E. General Formulae and Exemplary Compositions of the Invention

The APC-targeted antigens of the invention can
20 comprise one or more antigenic moieties, and likewise can comprise one or more targeting moieties. Moreover, these moieties can be functionally linked in several ways. For instance, if "T" represents a targeting moiety, and "Ag" represents an antigenic moiety, the APC-targeted antigens
25 of the present invention may be organized as follows:

$$Ag - T;$$
$$T - Ag;$$
$$T_1 - Ag - T_2;$$
$$T_1 - [Ag]_n - T_2 \text{ (wherein } [Ag]_n \text{ represents a}$$
30 multiplicity of antigens.

Examples of the general formulas set forth above include:

$$Ag - C5a \text{ agonist peptide;}$$
$$IFN\gamma \text{ peptide} - Ag;$$
35 $$IFN\gamma \text{ peptide} - [Ag]_n - C5a \text{ agonist peptide.}$$

Other representative compositions of the invention include:

MUC1 Class I binding epitope - C5a agonist C-terminal peptide

40 Murine or human IFNγ peptide - MUC1 Class I binding epitope

5   Murine or human IFNγ peptide - MUC1 tandém repeat

MUC1 Class I epitope - -C3dG peptide

SAA-K-Ahx - C5a C-terminal peptide (Ahx = εamino
hexanoic acid).

It will be appreciated by persons skilled in
10   the art that the APC-targeted activating antigens of the
invention may be adapted for inclusion of large or
complex antigens. This may be accomplished, for example,
by inclusion of a "spacer" (such as the K-Ahx spacer
moiety in the exemplary compound above) between the
15   antigen and the targeting moiety. Such chemical
modifications are familiar to biochemists.

II. Uses of APC-Targeted Activating Antigens

The APC-targeted activating antigens of the
20   present invention have broad potential for clinical
applications in humans and animals. As discussed above,
a significant impediment to the development of vaccines
and other immunotherapeutic agents is the apparent
inability of particular antigens to be readily taken up
25   and processed by antigen presenting cells. The
compositions of the invention facilitate the specific
delivery of an antigen to a population of antigen
presenting cells, whereupon the delivery mechanism (e.g.,
using as the targeting moiety a receptor ligand capable
30   of transducing a biological signal) simultaneously
activates the antigen presenting pathway. of the APC.
Thus, the present invention enables development of
vaccines and other immunotherapeutics that can
specifically target any peptide antigen or other
35   antigenic structure covalently attached to a ligand for a
receptor present on an antigen presenting cell. It is
believed that antigens linked to ligands that selectivity
bind to and activate a particular population of APCs can
not only generate an immune response, but can influence
40   the nature of the immune response that is generated.
Thus, immune responses that favor antibody, cellular, Th1

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or Th2 responses, respectiv ly, may be selectively
generated. Vaccines may also be developed with an array
of such targeting moieties thereby serving to target a
selected antigen or antigens to several populations of
5   APCs and simultaneously activate these and other cells
involved in various immune modulatory pathways.

The ability to generate either antibody or cell
mediated immune responses against different specific
antigens has broad general applicability, and it is
10   anticipated that the APC-targeted antigens of the
invention will be extremely useful for these purposes.
For example, antibody responses have been shown to be
capable of protecting against different viral or
bacterial infection, and antibodies are known to
15   inactivate different toxins or toxic compounds that may
affect the well being of humans or animals. Different
cell mediated immune responses can provide protection
against viral or other intracellular pathogens, and can
play a role in some anti-tumor responses. It is believed
20   that different antigen presenting cells and the context
in which these cells are stimulated to present antigen
(co-stimulation mediated by different ligand-receptor
interactions) are important factors determining the
nature of the above responses.
25   The targeted antigens of the present invention
should find particular utility in the development of
active specific immunotherapeutic agents (i.e., cancer
"vaccines") based on cancer-associated antigens. For
example, it has been hypothesized that induction of
30   strong cell-mediated immune responses (involving Th1
cells and/or cytotoxic T lymphocytes) would provide the
most effective protection against various forms of
cancer. A vaccination strategy utilizing the APC-
targeted antigens of the invention can be designed to
35   induce this type of response. In this regard, it is
known that stimulation with some cytokin s (IL-12, IFNγ)
can induce predominantly Th1 type responses over Th2 type

5   responses for certain antigens.

As a step toward developing anti-cancer
vaccines for clinical use, the compositions of the
invention can be used to advantage as research tools to
further explore the effect of stimulating a certain
10   population of APCs with a tumor antigen and determining
the effect on an anti-tumor immune response. To this
end, it should be noted that the present application
exemplifies targeted antigens comprising an epitope of a
particular tumor-specific antigen, Mucin-1.

15   Previous tumor vaccine formulations that aim to
immunize patients with compounds that are identical to
compounds already produced by tumors have proven to be of
limited value, probably because tumors that progress have
been selected for their lack of immunogenicity in their
20   respective host (e.g., the host is tolerant to existing
tumor antigens). Thus, one important challenge of
producing effective tumor vaccines is generating reagents
that counteract immunological tolerance to tumor-
associated antigens. One purpose of the APC-targeted
25   antigens described above is to induce in the immunized
individual a response against their tumor that is similar
to that seen in individuals undergoing allograft
rejection. In other words, the goal is to induce an
autoimmune reaction against the tumor that is capable of
30   destroying the tumor. The immunological parameters that
regulate tolerance to tumor antigens are not well
understood; nonetheless the compositions described herein
have the potential to counteract this tolerance and thus
induce specific immune responses that mediate tumor
35   rejection.

The targeted antigens of the present invention
will also find broad utility in the production of
antibodies for use as immunodiagnostic and
immunotherapeutic agents. For immunodiagnostic purposes,
40   antibodies are widely used in various quantitative and
qualitative assays for the detection and measurement of

5   biological molecules associated with diseases or other
pathological conditions. For reasons that often are not
well understood, it is sometimes difficult to generate
antibodies against certain biological molecules using
conventional means. The compositions of the present
10   invention provide an alternative means for inducing an
animal to produce antibodies against a weakly-antigenic
or non-antigenic substances. The utility of the
compositions of the invention in this regard is shown
clearly in Example 2, below, in connection with serum
15   amyloid A. The appearance and abundance of this protein
in the body is strongly correlated with systemic
inflammatory stress, which is a condition that is very
difficult to quantitate. It is believed that
quantitative assays for SAA levels would be an excellent
20   indicator of general, systemic inflammation; therefore it
would be of benefit to generate antibodies against the
protein in a non-human species. This protein has proved
particularly recalcitrant to the generation of antibodies
using conventional measures. As described in Example 2,
25   a targeted antigen comprising SAA conjugated to a C5a
peptide ligand produced a significant antibody response
in mice injected with the conjugated molecule. In a
similar fashion, targeted antigens comprising any weakly-
antigenic or non-antigenic component of interest could be
30   made and used to produce specific antibodies in
laboratory animals, for use as immunodiagnostic reagents.

        Antibodies for use as immunotherapeutic agents
can also be generated using the compositions of the
invention. As one example, there has been a great deal
35   of recent interest in developing reagents capable of
down-regulating or inhibiting the complement cascade to
modulate local and systemic inflammatory responses. To
this end, the C3a convertase, which is active early in
the cascade, could provide an ideal target for complement
40   inhibition. C3a convertase cleaves the peptide C3 into
two components, C3a and C3b, and therefore must be able

5   to access the cleavage site on C3 in order to accomplish
the result. Antibodies directed toward the C3a-C3b
cleavage site are expected to be effective in blocking
access of C3a convertase to the cleavage site, thereby
inhibiting this early step in the complement cascade.

10   Such antibodies may be generated using a targeted antigen
of the invention comprising, as the antigenic moiety, the
short peptide sequence comprising the C3a/C3b cleavage
site. The sequence could then be conjugated to an
appropriate targeting moiety, such as the C5a C-terminal

15   decapeptide agonists exemplified herein. Thus, the
compositions would be useful to generate an
immunotherapeutic agent (e.g., an antibody that blocks
the activity of C3a convertase) for treating an adverse
inflammatory condition.

20   The following examples are provided to describe
the invention in further detail. These examples are
intended to illustrate the invention in greater detail.
They are not intended to limit the invention in any way.

25   **EXAMPLE 1**
**Evaluation of Mucin Epitope (MUC1/C5a agonist) Conjugate
for Recruitment and Activation of Antigen Presenting
Cells (APCs) and Stimulation of an Immune Response in
Mice**

30   The C5a receptor is present on numerous antigen
presenting cells, including monocytes, macrophages,
dendritic cells, and other cell types. In this example,
a composite peptide comprising a mucin epitope (MUC1)
functionally linked to a ~~decapetide~~ decapeptide agonist analog of C5a

35   corresponding to the C-terminal effector region of the
natural factor was evaluated for its ability to activate
the APCs thereby stimulating an immune response in mice.
This evaluation is based on the known property of C5a
receptors to internalize and recycle in the antigen

40   presenting cell, thereby acting as ideal candidates for
delivering antigens to and simultaneously activating

5 signals in the APCs. Because C5a receptors are
particularly common on macrophages, monocytes and
dendritic cells, it is believed that the use of a C5a
agonist analog to bind C5a receptors will result in
preferential activation of these APCs.

10

i. **Abbreviations.** Except where noted, the single
letter designation for the amino acid residues is used:
alanine is A; arginine is R; asparagine is N; aspartic
acid is D; cystine is C; glutamine is Q; glutamic acid is

15 E; glycine is G; histidine is H; isoleucine is I; leucine
is L; lysine is K; methionine is M; phenylalanine is F;
proline is P; serine is S; threonine is T; tryptophan is
W; tyrosine is Y; and valine is V. Upper case letters
represent the L-amino acid isomer and lower case the D-

20 isomer.

ii. **Peptide synthesis, Purification and
Characterization.** The following peptides were
synthesized according to standard solid-phase
methodologies on an Applied Biosystems (Foster City, CA)

25 model 430 A peptide synthesizer and characterized as
previously described (7):

(1) The antigenic "juxta-membrane" (JM) epitope of
the human mucin-1 (MUC1), YKQGGFLGL (SEQ ID NO:6);

(2) The C5a C-terminal decapeptide agonist analog,

30 YSFKPMPLaR (SEQ ID NO:1);

(3) The composite peptide YKQGGFLGLYSFKPMPLaR (SEQ
ID NO:2), in which the JM epitope is positioned toward
the amino terminus and the C5a peptide is positioned
toward the carboxyl terminus; and

35 (4) The composite peptide YSFKPMPLaRKQGGFLGL (SEQ
ID NO:5), in which the JM epitope of MUC1 is positioned
toward the carboxyl terminus and the C5a analog is
positioned toward the amino terminus.

Peptide 3 retains C5a biological activity,

40 whereas peptide 4 does not because the biologically
important carboxyl terminal end of the C5a analog is

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blocked by the presence of the mucin epitop . As such,
peptide 4 serves as a control to determine the importance
of the C5a biological activity to the effectiveness of
these peptides for immunization purposes.

5   Syntheses were performed on a 0.25 mmol scale
on O-hydroxymethylphenoxymethyl polystyrene (HMP) resins
(0.88 meq/g substitution). N^{α}-amino groups were protected
with the base-labile 9-fluorenylmethyloxycarbonyl (Fmoc)
group. Side-chain functional groups were protected as
10   follows: Arg (Pmc or 2,2,5,7,8-pentamethylchroman-6-
sulfonyl); Asp (Ot-butyl ester); Cys, Gln & His (Trt or
trityl); Lys (Boc or t-butyloxycarbonyl); Ser & Tyr (t-
butyl). Synthesis was initiated by the *in situ* coupling
of the C-terminal residue (N^{α}-Fmoc-L-Arg(Pmc)) to the HMP
15   resin in the presence of excess N-N'-
dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole
(HOBT) with 4-dimethylaminopyridine (DMAP) as a coupling
catalyst. Peptide chain elongation was accomplished by
repetitive Fmoc deprotection in 50% piperidine in NMP
20   followed by residue coupling in the presence of 2-(1H-
benzotriazol-1-yl)-1,1,3,3-tetramethyluronium
hexafluorophosphate (HBTU).

          Side-chain deprotection and cleavage from the
resin were achieved in a single step acetolysis reaction
25   by stirring the peptide-resin in a solution of 84%
trifluoroacetic acid (TFA), 6% phenol, 2% ethanedithiol,
4% thioanisole, and 4% water for 1.5 hr at room
temperature. Free peptide was precipitated from this
solution by adding cold diethyl ether. The mixture was
30   filtered through a scintered glass Buchner funnel (medium
porosity) and the peptide/resin washed twice with cold
ether to remove the thiol scavenger. The peptide was
extracted by swirling the peptide/resin in the funnel
with 20-30 ml aliquots of 10% acetic acid followed by
35   filtration. The extraction aliquots were combined,
frozen, and lyophilized to yield the powdered form of the
crude peptide.

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Peptides were purified by pr parative and
analytical reverse-phase HPLC on columns pack d with C_{18}-
bonded silica. The details of this procedure have been
described by (4). All peptides were characterized by
5   amino acid compositional analysis and fast atom
bombardment mass spectrometry (FAB-MS).

iii. **Animal Models.** The strains of mice used for
this example were inbred females 6 to 12 week old
C57B16($H-2^b$) and Balb/c ($H-2^d$), which were obtained from
10   Jackson labs (Bar Harbor, Maine). These two strains
which differ in H-2 haplotypes, were used in this example
to demonstrate that the observed antibody responses were
not a result of the selection or creation of an unique
immunogenic epitope characteristic of the sequence of the
15   proteins of the MHC class I and class II molecules
important for antigen processing in one mouse strain or
another. The MUC1 peptide selected for these studies
contained a motif that may bind to the $H-2K^b$ molecule of
the C57B16 mice; therefore, a strain of mouse that lacked
20   this class I molecule binding motif (Balb/c) was also
studied to determine the relative contribution of the
class I binding motif to the antigen presentation
properties of these peptides.

iv. **Immunization protocol.** Preimmune sera were
25   obtained from mice, which were subsequently immunized
intraperitoneally with 100 μg of the indicated peptide
with RIBI adjuvant (MPL+TDM+CWS) (Sigma Immunochemicals).
Animals were boosted twice at two week intervals using
the same injection procedure. Sera were obtained
30   following three immunizations (at 6 weeks).

v. **Analysis of serum antibody responses.** For
radioimmunoassay (RIA), anti-peptide antibodies were
determined, before and at different time points after
immunization, in 96-well microtiter plates (Dynatech
35   Laboratories, Inc.). Plates were coated with 50 μl of a
100 μg/ml appropriate peptide in phosphate-buffered
saline (PBS) pH 7.4 solution overnight at 4°C. The wells

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were blocked by incubation with 5% dry milk in PBS pH 7.4
for at least two hours. Anti-peptide antibody titers
were determined using serial dilutions of sera. The sera
were diluted with PBS containing 0.05% Tween-20, pH 7.4

5   (washing buffer) and 50 μl of each dilution was incubated
at 37°C for 1 hour. The wells were then drained, washed
4 times with PBS-Tween and 50 μl of ^{125}I-goat anti-mouse Ab
(1 - 2 x 10^4 cpm/well) was added and incubated for 1 hr at
25° C. After washing, specific radioactivity was recorded

10   in a gamma counter (1272 CliniGamma, LKB).
        Anti-peptide antibody isotype titers were
determined by enzyme-linked immunosorbent assay (ELISA)
carried out in 96-well microtiter plates. The plates
were coated with 100 μg/ml of appropriate peptide in PBS,

15   pH 7.4, and incubated overnight. The wells were blocked
with 5% dry milk in PBS pH 7.4 for at least two hours.
Anti-peptide titers were determined using serial
dilutions of sera as described above. After the plates
were washed 4 times, 50 μl of a 1:100 dilution of rabbit

20   anti-mouse IgA, IgG1, IgG2a, IgG2b, IgG3 and IgM (Zymed)
was added to each well and incubated at 25°C for 1 hour.
The plates were washed 4 times with washing buffer and 50
μl of 1:500 goat anti-rabbit conjugated to peroxidase
(Zymed) was incubated at 37°C for 1 hour. Again, the

25   plates were washed 4 times with washing buffer and bound
enzyme was detected by the addition of 50 μl 1 mg/ml p-
nitrophenyl phosphate (Sigma) in 10% diethanolamine
(Sigma) pH 9.4. The reaction was stopped by the addition
of 50 μl of 0.5 M sodium hydroxide and absorbance values

30   (A$_{405}$) were determined on Titertek Multiskan (Flow
Laboratories, Irvine, Scotland).
        vi. **Experimental groups.** Experimental groups were
as follows:
                Group A.   mice immunized with peptide (1)

35   Group B.   mice immunized with peptide (2)
                Group C.   mice immunized with peptide (1) plus
                           peptide (2)

5   Group D. mice immunized with peptide (3)
           Group E. mice immunized with peptide (4).

           The results of the experimental protocols are
       set forth in Figures 1 and 2. As can be seen in the
10   Figures, the mice in Groups A, B, C and E produced no
       appreciable increase in antibody response to inoculation
       with MUC1 epitope (Group A), C5a agonist peptide (Group
       B), MUC1 epitope combined with, but not conjugated to,
       C5a agonist peptide (Group C), or MUC1 epitope conjugated
15   to the C5a agonist peptide at its C-terminus, rather than
       its N-terminus (thereby blocking C5a biological activity)
       (Group E). Only mice inoculated with the MUC1
       epitope/C5a agonist peptide conjugate of the present
       invention (Group D) generated an appreciable antibody
20   response. Furthermore, this stimulation was significant.
       It is clear from these results that inoculation with the
       conjugated MUC1 epitope/C5a agonist peptide was far more
       efficient in stimulating a general immune response (i.e.,
       production of antibodies) than was inoculation with
25   either peptide alone, or both peptides together, but not
       conjugated, or peptides conjugated in the opposite
       orientation.
           There are several significant conclusions that
       can be drawn based on these results. The fact that both
30   Balb/c and C57B16 mice showed antibody responses to
       peptide 3 suggests that the antigen presenting effect is
       not restricted by MHC haplotype. The fact that immune
       responses were not produced to peptide 4, or to mixtures
       of peptide 1 and 2, but that substantial responses were
35   produced to peptide 3, suggest that the effect is
       mediated by the C5a moiety of the peptide and that the
       immune response results from the simultaneous delivery of
       antigen peptide and C5a mediated activation signals to
       antigen presenting cells.
40   The isotypes of the anti-peptide antibodies
       produced in the immunized mice were determined (Figure 3)

5   and were found to consist of IgM, IgG2a, and IgG2b. This
     suggests that the immunogenic peptide is producing T
     cell-dependent responses, which generally require antigen
     processing and presentation. Data presented in Figure 4
     show that the antibody response to peptide 3 includes a
10   high percentage of antibodies that are specific for the
     MUC1 epitope that was the antigen moiety of these
     studies.

15   EXAMPLE 2
                 Evaluation of Serum Amyloid A/C5a Peptide
                 Conjugates for Recruitment and Activation of
                 APCs and Stimulation of Immune Response in Rats
                     Serum amyloid A is an acute-phase stress
20   response protein generated by the liver. Along with
     other acute phase proteins, SAA is secreted in response
     to systemic inflammatory stress as a protective measure.
     SAA is of interest because it appears to be an excellent
     indicator of general, systemic inflammation, which is a
25   phenomenon that is very difficult to quantitate. Because
     serum levels of SAA have been observed to parallel the
     rise and fall of the systemic inflammatory response,
     quantitation of serum levels of this peptide would
     provide an effective means of assessing inflammation.
30   One way to accomplish this is to develop antibodies
     against SAA that could be used for quantitation such as
     in an ELISA assay. However, SAA has been particularly
     recalcitrant to the generation of antibodies against it.
     In this example, an evaluation was made of the ability of
35   SAA conjugated to a C5a C-terminal analog (as described
     in Example 1) to activate antigen producing cells and
     produce an antibody response in rats.

         i.   Production and preparation of proteins and
40   peptid s. The C-terminal C5a analog K-Ahx-YSFKPMPLaR (SEQ ID
     NO:8) (AhX is aminohexanoic acid, which is a linear aliphatic

spacer moiety) was produced as described in Example 1. The aliphatic spacer moiety was included to separate the critical receptor-binding C5a analog from the bulky protein to be attached to the amino terminus.

Serum amyloid A was conjugated to the C5a peptide analogs according to the following method. SAA (100 μg) was reacted with a 50-fold molar excess of a water soluble carbodiimide, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide methiodiide (EDC), in 200 μl of phosphate buffered saline, pH 7.5, at room temperature for 30 minutes. A 50-fold molar excess of the peptide (K-Ahx-YSFKPMPLaR) (SEQ ID NO:8) and a 100-fold molar excess of a base diisopropylethyl amine (DIEA) were added to this solution. Water was added to the solution to bring the reaction mixture to a volume of 400 μl. This solution was stirred overnight at room temperature and then lyophilized to a dry powder. The powder was diluted to the appropriate volume with water to generate the stock mixture used for inoculating the animals.

ii. Experimental protocols. Rats were injected intraperitoneally with an inoculant comprising the SAA/C5a peptide conjugates in phosphate-buffered saline with or without RIBI adjuvant. Booster injections were given two and five weeks after the initial injections. The rats were sacrificed seven weeks after the initial injection and anti-mucin antibody production was assessed from the serum titers, as described in Example 1.

Significant anti-SAA antibody was produced from both groups of rats, whether or not RIBI adjuvant was included in the inoculation. As visualized by gel electrophoresis and autoradiography of anti-SAA antibody eluted from the plate assays, it appeared that anti-SAA antibody titers were essentially equivalent, or slightly higher, in rats inoculated with SAA/C5a peptide conjugate in the absence of RIBI adjuvant as compared to the same

5   inoculation without the adjuvant. Thus, antigenic
     conjugates comprising the C5a peptide analog are useful
     for generating antibodies against large proteins, as well
     as against smaller peptide fragments, such as those
     described in Example 1. Moreover, the successful
10   generation of anti-SAA antibodies utilizing this method
     is particularly promising for purposes of producing
     antibodies against weakly- or non-antigenic peptides or
     proteins.

EXAMPLE 3

15   **Production and Characterization of Site-Directed**
     Neutralizing Antibodies Specific for a Peptide κR(33-52)
     from the Predicted Amino-Terminal Region of the
     Human Kappa Receptor

     Receptors for human opioid peptide hormones have
20   been described on numerous cell types. The receptors for
     μ, κ, and δ ligands have recently been cloned from
     genomic and cDNA libraries derived from normal tissue and
     cell lines. Considerable homology exists among the μ,
     κ, and δ receptors, except for the N-terminal regions of
25   the receptors. The N terminal region of the human kappa
     receptor (amino acid residues 1-100) is relatively
     hydrophilic and would be predicted to be exposed on the
     surface of the cell membrane. A 20 residue peptide
     [κR(33-52)], was chosen and used to raise a site directed
30   peptide specific polyclonal antibody (5).

     The method of production of a polyclonal antiserum
     in rabbits using the molecular adjuvant, C5a-agonist
     peptide conjugated to the κR epitope is set forth below.
     The binding specificity and biological activities of the
35   resulting polycolonal antiserum raised to the predicted
     extracellular region of the human kappa receptor (κR) are
     also described below.

     i.   **Construction of Targeted-Immunogen.** A peptide
     construct consisting of the κR(33-52)
40   (FPGWAEPDSNGSAGSEDAQL) (SEQ ID NO:9) covalently attached
     to the N-terminal end of a conformationally biased, C5a

complement fragment agonist analogue peptide (YSFKPMPLaR)
(SEQ ID NO:1) was synthesized according to the methods in
Example 1 and as previously reported (7).

    ii. **Preparation of anti-κR(33-52) Antiserum and
Peptide-Specific ELISA.**   Rabbits were immunized s.c. with
500 μg of FPGWAEPDSNGSAGSEDAQLYSFKPMLaR construct (SEQ ID
NO:10) in complete Freund's adjuvant (GIBCO, Grand Island,
NY) on day 0 followed by booster injections on days 30 and
60 in incomplete Freund's adjuvant. Serum was collected
starting 75 days after the initial immunization.

    The presence of anti-peptide antibody was determined
by using a peptide specific ELISA utilizing the free
κR(33-52) peptide as previously described (8). Anti-κR(33-
52) and normal rabbit γ-globulin (RGG) were purified by
protein A Sepharose chromatography (Sigma) (8) prior to
use.

    iii. **Cells and culture conditions.** The
neuroblastoma cell SK-N-SH (HTB 11), ductal breast cell
carcinoma T47D (HTB 133) , Jurkat T cell leukemia, (TIB
152), U937 histolytic lymphoma (CRL1593), THP 1 human
monocyte (TIB 202), EBV-transformed B cells SKW 6.4 (TIB
215) and CESS (TIB 190) (American Type Culture
Collection, Rockville, MD) were cultured in DMEM or RPMI
1640 supplemented with 10% fetal calf serum, 25 mM HEPES,
1 mM L-glutamine, 2 mM Na pyruvate, 50 U penicillin and
50 μg/ml streptomycin. The human neuronal precursor
cells NT2 (Stratagene, La Jolla, CA) were cultured in
Opti-MEM (Gibco) supplemented as above. All cultures
were incubated at 37° C in a humidified chamber with 7.5%
CO_2.

    Peripheral blood derived mononuclear cells were
obtained from healthy male and female volunteers,
isolated by Ficoll-Hypaque(tm) density gradient
centrifugation and enriched for macrophage by adherence
to plastic.

5 **iv. Flow Cytom try.** Single-color flow cytometry
analysis of cells (1 X 10^6) in PBS containing 1% bovine
calf serum and 0.1% sodium azide (staining buffer) were
preincubated 30 minutes at 4° C in the presence of 20%
normal human serum. The cells were washed and incubated
10 with anti-κR(33-52) or RGG for 30 minutes at 4° C, washed
and labeled with PI-conjugated donkey (Fab')2 fragments
of antirabbit IgG (Zymed, S. San Francisco, CA) for 30
minutes at 4° C (8). For dual color analysis FITC-
conjugated anti-CD3 or anti-CD14 (Pharmingen, San Diego,
15 CA) were also included in the second step. Cells (1 x
10^4) were analyzed on a FACScan (Becton Dickinson,
Mountain View, CA) and data were analyzed with the Cell
Quest software as previously described (8).

20 **v. Measurement of cell proliferation.** Peripheral blood
mononuclear cell (PBMC) were pulsed on day 2 of culture
with ^{3}H-thymidine and 18 hours later the cells harvested
on glass fiber filters and processed for scintillation
counting. Experiments were performed three times and
25 each sample done in triplicate.

vi. Measurement of IgG Secretion. Relative IgG levels in
culture supernatants were determined by indirect ELISA as
previously described (9). Supernatants from PBMC cultures
30 were collected after 10 days and assayed for the presence of
IgG. Numbers represent the mean CPM +/- SD from triplicate
samples. Experiments were performed at least three times.

vii. Characterization of Anti-κR Peptide Antiserum. Serum
35 from rabbits immunized with the κR(33-52)YSFPMPLaR
construct (SEQ ID NO:10) and normal rabbit serum were
assayed for the ability to recognize plate bound κR(33-52)
(SEQ ID NO:9) in ELISA. The results show that serum from
rabbits immunized with the κR(33-52)YSFPMPLaR construct
40 (SEQ ID NO:10) bound free κR(33-52) peptide (SEQ ID NO:9)
in a dose dependent fashion. The titer was approximately

5   10^5. In contrast, serum from unimmunized rabbits failed to
bind this peptide. Serum samples from immunized and
unimmunized rabbits were subjected to protein A-Sepharose
chromatography and the column eluates were assessed for
κR(33-52) (SEQ ID NO:9) specific antibody. The results
10   indicate that protein A-purified antibody derived from
rabbits immunized with the κR(33-52)YSFPMPLaR construct
(SEQ ID NO:10) binding to free κR(33-52) (SEQ ID NO:9) was
detectable at antibody concentrations less than 0.1 ng/ml.
In contrast, RGG failed to bind the free peptide. The
15   results from multiple bleedings indicated that the ED_{50}
titer ranged between 1-10 ng/ml. These results indicate
that rabbits immunized with κR(33-52)YSFPMPLaR (SEQ ID
NO:10) contained high titer, κR(33-52) peptide specific
antibody.

20

**viii.   Binding of anti-R (33-52) antibody to cells
expressing human κR.** To determine whether the polyclonal
anti-κR(33-52) antibodies bound to cells expressing the
25   κR, a variety of mononuclear cell lines and normal human
mononuclear cells were first assayed for the presence of
the κ receptor specific mRNA by RT-PCR. RNA samples
isolated from neuronal cell lines NT2, U937, Jurkat,
T47D, normal human PBMC, and enriched human macrophage
30   were subjected to RT-PCR analysis with 5' sense and 3'
antisense primers specific for the 3' region of the
cloned κR and B-actin. All of the cell lines or cell
fractions, except for the T47D cell line, were positive
for the κ-receptor specific PCR product, as expected
35   based on the primer sequences used (5).

Experiments were performed to determine whether
anti-κR(33-52) bound to cells expressing κR specific
mRNA. The results of single color flow cytometric
analysis for several cell lines are shown in Table 2.
40   Flow cytometric measurements were conducted with human
cell lines representative of macrophage (U937), T

- 42 -

lymphocytes (Jurkat), and B lymphocytes (SKW 6.4 and
CESS). The results indicate that anti-κR(33-52) bound all
three cell types. Anti-κR(33-52) bound to U937 cells to
the greatest extent (MFI=231) compared to normal RGG

5   (MFI=38). As used herein MFI refers to mean fluorescence
intensity. Comparison of anti-κR(33-52) and RGG binding
to the Jurkat line indicated approximately a 3-fold shift
in MFI (MFI=18 vs. MFI=6). Similar results were obtained
with the two B lymphocyte-like cell lines (SKW 6.4 and

10   CESS). Comparison of anti-κR(33-52) and RGG binding to
the SKW 6.4 line indicated approximately a 3-fold shift
in MFI (MFI=19 vs. MFI=6). The neuronal cell line was
also specifically bound by the anti-κR(33-52) as
indicated by a 3-fold shift in the MFI over the RGG.

15   Finally, based on the lack of expression of κR-specific
mRNA from the human breast carcinoma cell line (T47D),
this cell line was assessed for its ability to bind to
anti-κR(33-52) by flow cytometric analysis. The lack of a
κR expression on T47D cells was confirmed by the fact

20   that anti-κR(33-52) and RGG bound to these cells in an
almost identical fashion. As a positive control,
anti-κR(33-52) and RGG were assessed for their ability to
bind to an additional human macrophage-like cell line
(THP 1). Comparison of anti-κR(33-52) and RGG binding to

25   this cell line resulted in a significant shift in MFI
(MFI=190 vs. MFI=8). These results confirm the
specificity of anti-κR(33-52) for the human κR.

30

35

40

Table 1
Selected cell type binding of anti-κR(33-52)
antibodies produced in rabbits immunized with
C5a-agonist peptide conjugated to the κR(33-52)
sequence as assessed by single channel color
flow cytometric analysis.

Cell Line	Cell Type	Mean Channel Intensity	
		RGG	anti-κR Ab
NT2	Neuronal	9	19
U937	Macrophage	38	231
Jurkat	T-lymphocyte	6	18
SKW 6.4	B-lymphocyte	6	19
CESS	"	<10	>10
Controls			
T47D (negative)	Human Breast Carcinoma	~3	~3
THP1 (positive)	Macrophage	8	190

Analysis of intact human PBMC indicated that
these cells express mRNA for a "κ-like" R (5). Dual
color flow cytometric analysis was utilized to assay for
the binding of anti-κR(33-52) to normal human macrophage
(CD14+) and T lymphocytes (CD3+). It was observed that
both macrophage and T lymphocytes bound anti-κR(33-52)
antibody. Anti-κR(33-52) and RGG were assessed for
binding to CD14+ PBMC. The results indicate that
anti-κR(33-52) bound CD14+ cells with a 15-fold increase
compared to normal RGG (MFI=320 vs. MFI=21).
Anti-κR(33-52) was also found to bind CD3+ cells (MFI=19
vs. RGG MFI=3) albeit less than CD14+ cells. These
results indicate that anti-κR(33-52) binds normal
PBMC-derived mononuclear cells as well as mononuclear
cell lines, which express κR-specific mRNA.

**ix. Neutralization of U50,488H-mediated suppression of
lymphocyte proliferation by anti-κR(32-52) antibody in
vitro.** The results of published studies have shown that

opioid peptide-induced regulation of *in vitro* immune responses can occur via specific receptor-ligand interactions. More specifically, it has been shown that the κR-selective agonist U50,488H is capable of suppressing SAC-induced lymphocyte proliferation by human PBMC cultures (6). The inhibition of lymphocyte activation by U50,488H has also been shown to be reversed by the κR-selective antagonist nor-BNI. To determine whether anti-κR(33-52) was capable of acting as an κR selective antagonist and neutralizing U50,488H-mediated suppression, PBMC cultures were preincubated with various concentrations of protein A purified anti-κR(32-52) prior the addition of SAC and U50,488H. U50,488H suppresses SAC-induced lymphocyte proliferation in a dose dependent fashion (5). Maximal suppression was obtained when U50,488H was used at a concentration of 10^{-6} M. PBMC cultures were preincubated with various concentrations of anti-κR(33-52) (1-50 μg/ml), followed by the addition of U50,488H plus SAC, and proliferation measured on day 3 of culture. Anti-κR(33-55) was found to neutralize U50,488H-mediated suppression of SAC-induced lymphocyte proliferation in a dose dependent fashion. In contrast, identical concentrations of normal RGG failed to inhibit κR selective agonist mediated immunosuppression.

Since SAC has been shown to induce both T and B lymphocyte proliferation, similar experiments were conducted with the T cell mitogen PHA. Anti-κR(33-52) was also able to neutralize the ability of U50,488H to suppress mitogen-induced T cell proliferation. U50,488H (10^{-6} M) suppressed PHA-induced T cell proliferation by 85%. This suppression was reversed by preincubating the cells with anti-κR(33-52). Preincubation of PBMC with normal RGG failed to block U50,488H-mediated suppression of T cell proliferation.

Anti-κR(33-52) does not appear to directly modulate lymphocyte proliferation. The co-culture of PBMC with anti-κR(33-52), in the absence of mitogen, failed to

stimulate the cells above the media control. Moreover, the
combination of anti-κR(33-52) and PHA or SAC did not
result in increased cell proliferation compared to PBMC
cultures receiving mitogen only.

**x. Neutralization of U50,488N-mediated suppression of
IgG synthesis by anti-κR(32-52) antibody in vitro.** In
addition to lymphocyte proliferation, U50,488H is a potent
inhibitor of SAC-induced IgG synthesis in human PBMC
cultures (6). To determine whether anti-κR(32-52) was
capable of neutralizing the suppression of IgG synthesis,
PBMC were preincubated with anti-κR(32-52) followed by the
addition of U50,488H and SAC, and IgG levels measured on
day 10. Results indicate that U50,488H at 10^{-8} M and 10^{-7} M
inhibited IgG synthesis by 67% and 85% respectively (5).
The inclusion of anti-κR(32-52) in culture was found to
neutralize suppression of SAC induced IgG synthesis in a
dose dependent manner. In contrast, similar
concentrations of normal RGG failed to neutralize the
observed suppression.

To assess the specificity of anti-κR(32-52)
antibody, PBMC were incubated with specific antibody or
RGG followed by co-culture with U50,488H or the μ receptor
selective agonist (DAGO) and IgG production measured by
ELISA. The results indicate that, whereas, anti-κR(32-52)
neutralized U50,488H-mediated inhibition of SAC-induced
IgG synthesis, anti-κR(32-52) was unable to neutralize
DAGO-mediated suppression of IgG synthesis.

These results indicate that in addition to
binding lymphocytes and macrophage, anti-κR(32-52) is
capable of neutralizing the ability of a κR selective
agonist (U50,488H), but not a μR selective agonist (DAGO).
Additionally the antibody demonstrated significant
inhibition of both lymphocyte proliferation and
differentiation to antibody synthesis. These results
further demonstrate the specificity of anti-κR(33-52) for

5   the human kappa receptor.

As can be seen from the antibody binding data
presented above, the site directed polycolonal antibodies
raised in rabbits using the C5a-agonist form of the
molecular adjuvant conjugated to the κ receptor sequence

10   were capable of binding to normal human cells and cell
lines expressing mRNA specific for the human κ receptor.
Flow cytometric analysis of a neuronal cell line (NT2),
normal blood-derived CD14+ monocytes, monocyte-like cell
lines (U937 and THP1), normal blood derived CD3+ T cells

15   and a T cell line (Jurkat), and human B cell lines (SKW6.4
and CESS) revealed that the cells were all bound by anti-
κR(33-52) in a specific manner. The anti-κR(33-52) did not
bind to a cell line determined not to express mRNA for the
human κ receptor.

20   Anti-κR(32-52) was found to specifically
neutralize κR-selective agonist (U50,488H)-mediated
inhibition of lymphocyte activation. The antiserum was
found to neutralize, in a dose dependent manner,
U50,488H-mediated inhibition of: 1) SAC-induced lymphocyte

25   proliferation; 2) PHA-induced lymphocyte proliferation
and; 3) SAC-induced IgG synthesis. In contrast,
DAGO-mediated suppression of SAC-induced IgG production
was not affected by anti-κR(32-52). These results suggest
that this site directed polyclonal antiserum specifically

30   interacts with the human κR on PBMC. The results
presented indicate that polyclonal anti-κR(32-52)
antibodies interact with the exposed N-terminal region of
the κR. While this antiserum effectively blocked U50,488H
-mediated lymphocyte activation, it did not activate

35   macrophage or lymphocytes.

While anti-κ opioid receptor antibodies are
exemplified above, conjugation of C5a agonist peptide to
peptides corresponding to μ and Δ specific peptides has
resulted in the successful generation of specific

40   antibodies to the μ and Δ epitopes.

EXAMPLE 4.

Comparison of Immunog nicity of Epitope-C5a agonist constructs with pitope-KLH conjugat s.

The following experiment was performed in order to compare the potency of the molecular adjuvant of the present invention with a widely used method for enhancing the immune response to peptide epitopes. The objective was a direct comparison of the response to a construct of MUC1 epitope-C5a agonist and the same epitope conjugated to keyhole limpet hemocyanin (KLH) in mice. The results are summarized in Table 2.

Table 2

MUC1 Specific Ab Isotype Titers
Produced with Different Immunogens.

Ab Isotypes and Titers[a]

	IgA	IgG1	IgG2a	IgG2b	IgG3	IgM
YKQGGFLGLYSFKPMPLaR[b] (SEQ ID NO:2)	0	0	1260 (5/5)	1780 (5/5)	0	6310 (5/5)
YKQGGFLGL-KLH[c] (SEQ ID NO:6-KLH)	0	100 (2/5)	0	0	0	5010 (4/5)

[a] Sera were screened against MUC1 peptide and mean titer values of responders are shown. Parentheses indicate the number of responders. Ab titer is defined as the sera dilution within the linear range at which specific reactivity is lost.

[b] Five C57BL6 mice were immunized and boosted with YKQGGFLGLYSFKPMPLaR (SEQ ID NO:2) and sera were obtained as indicated in the Material and Methods section. Standard error of responder titer values was less than 32% for all isotypes.

[c] Five C57BL6 mice were immunized and boosted with YKQGGFLGL-KLH (SEQ ID NO:6 conjugated to KLH) and sera were obtained as indicated in the Materials and Methods section. Standard error of responder titer values was less then 25% for IgM and less than 40% for IgG1.

A similar experiment was performed in rabbits. The immunogens used in rabbits were the κ- and μ- opioid

5   receptor epitopes, FPGWAEPDSNGSAGSEDAQL (SEQ ID NO:9) and
       GDLSDPCGNRTNLGGRDSL (SEQ ID No:11), respectively. The
       serum antibody titer and antibody subtypes produced in
       rabbits injected with the two compositions containing the
       different immunogens were compared.
10

              i.   **Peptide conjugates.** In one instance the
       epitopes were conjugated to KLH via a lysine residue added
       synthetically to the N-terminus of the epitope along with
       an alanine residue which acted as a spacer. In this
15   experiment, glutaraldehyde was used to effect conjugation.
        In the another case, the epitopes were linked to the N-
       terminal end of the C5a agonist YSFKPMPLaR (SEQ ID NO:1)
       using the solid phase peptide synthetic methodologies
       described above in example 1.
20   ii.   **Immunization protocol for rabbits.** Rabbits were
       immunized s.c. with 500 μg of either the epitope-KLH or
       the epitope-YSFKPMPLaR (epitope-SEQ ID NO:1) constructs in
       compete Freund's adjuvant (GIBCO, Grand Island, NY).
       Booster injections were administered on days 30 and 60 in
25   incomplete Freund's adjuvant. Serum was collected
       starting at day 60 post-immunization.
              iii. **Antibody determination.** The presence of rabbit
       IgG specific for the peptide epitopes was determined by
       ELISA as previously described (8).
30   Rabbits immunized with the epitope-C5a agonist
       generated high titer IgG Abs specific for the opioid
       receptor peptide epitopes. The rabbits immunized with the
       opioid receptor epitopes conjugated to the carrier protein
       KLH also produced high titer antibodies specific epitopes
35   to which they were injected. These results demonstrate
       that the decapeptide C5a-agonist was as effective as the
       large molecular weight protein, KLH, conjugated to the
       epitope at inducing specific anti-peptide antibodies in
       non-rodent species.
40

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While certain preferred embodiments of the present invention have been described and specifically exemplified above, it is not intended that the invention be limited to such embodiments. Various modifications

may be made to the invention without departing from the
scope and spirit thereof as set forth in the following
claims.

5